ORIGINAL ARTICLE

Detection of pancreatic cancer using serum protein profiling

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Abstract

Background: Currently, no suitable biomarkers for the early detection of pancreatic cancer (PC) are available. Proteins present in the serum could reflect a state of the disease. In this study, these profiles as a diagnostic marker for PC were evaluated.

Methods: Serum samples were obtained from PC patients ($n = 50$ calibration set, $n = 39$ validation set) and healthy volunteers ($n = 110$ and $n = 75$ respectively) according to a uniform standardized collection and processing protocol. For peptide and protein isolation, automated solid-phase extraction (SPE) with Weak Cation Exchange (WCX) magnetic beads (MB) was performed using a 96-channel liquid handling platform. Protein profiles were obtained by mass spectrometry (MS) and evaluated by linear discriminant analysis with double cross-validation.

Results: A discriminating profile for PC has been identified, with a sensitivity of 78% and a specificity of 89% in the calibration set with an area under the curve (AUC) of 90%. These results were validated with a sensitivity of 74% and a specificity of 91% (AUC 90%).

Conclusion: Serum profiles of healthy controls and PC can be discrimated between. Further research is warranted to evaluate specificity and whether this biosignature can be used for early detection in a high risk population.

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Introduction

Although pancreatic cancer (PC) has an annual incidence of only 8.2 cases per 100 000 males and of 5.4 cases per 100 000 females, it is the fifth (male) and fourth (female) leading cause of cancer death in developed countries.[1] Patients with PC have an extremely poor prognosis with an overall 5-year survival rate of less than 5%.[2] When a surgical resection is possible, 5-year survival rates increase to approximately 25%, but unfortunately most tumours are at an advanced stage when diagnosed.[3,4] Delays in diagnosis are often caused by the lack of specific symptoms for early cancers, such as pain, jaundice and weight loss. Biomarkers might be an additional tool for diagnostics next to currently available imaging techniques. The mostly studied available clinical serum biomarker carbohydrate antigen 19-9 (CA19-9) has a sensitivity of 80% and a specificity of 90% but misses the appropriate sensitivity and specificity for small, resectable cancers.[5] Moreover, CA19-9 is often elevated in benign cholangitis, pancreatitis and other cancers, and therefore lacks the specificity for detecting potentially curable lesions. At this moment, the use of CA19-9 is only recommended for follow-up. Currently, only imaging techniques such as ultrasound (transcutaneous or endoscopic), computed tomography (CT) scan, endoscopic retrograde cholangiopancreatography (ERCP), magnetic resonance imaging (MRI) and MR cholangiopancreatography (MRCP) are used for the diagnosis and staging of the pancreatic disease.

Chronic pancreatitis could mimic PC at diagnostics and hampers patient selection for a pancreaticoduodenectomy. For these patients a new biomarker that discriminates between pancreatitis and cancer could be of great value.

It has been estimated that 5% to 10% of PC cases are associated with an inherited predisposition. Tumour syndromes associated

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with an increased risk of PC include Peutz–Jeghers syndrome, familial atypical multiple mole melanoma (FAMMM), hereditary breast cancer (BRCA2 mutation carriers) and possibly Lynch syndrome. The lifetime risk of PC varies between 5% in BRCA2 mutation carriers and 36% in patients with Peutz–Jeghers syndrome.[6–9] For this patient group, early detection is of paramount importance as the prognosis is usually poor when diagnosis follows symptoms. No studies for the early detection in this specific high-risk group using CA19-9 have been performed. At this time this is only possible through imaging surveillance.[10] Therefore, there is an urgent need for new and better biomarkers for PC.

A sensitive and specific option could be the use of proteomic serum biomarkers. During transformation of a normal cell into a neoplastic cell, distinct changes occur at the protein level which may affect cellular function.[11] Therefore, proteins are considered promising targets for biomarker discovery. Mass spectrometry (MS) has become the method of choice for protein analysis in serum.[12,13] Provided standardized sample workup, MS measurement, data processing and evaluation, peptide and protein profiles are highly reproducible.[14] With respect to speed and automation strategies needed for high-throughput screening, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS workflows remain unrivalled.[12,15,16] As human body fluids such as serum are highly complex a suitable 'clean-up' procedure is required.[17] Based on the physicochemical properties of protein separation techniques, magnetic beads (MB) have been functionalized accordingly [e.g. Weak Cation Exchange (WCX)]. These beads are not only suited for clean-up, but also enrich subsets of peptides and proteins and can thus contribute to the sensitivity of the assay.[18] The present serum peptide/protein capture procedure has been fully automated with a liquid handling robot, such as a 96-channel Hamilton STARplus® platform (Hamilton, Bonaduz, Switzerland). This ensures reproducibility and allows high-throughput screening which is essential for large-scale disease profiling studies.[19,20] In the last decade, multiple studies have been carried out using a magnetic bead-based method for offline serum peptide/protein capture and MALDI-TOF-MS readout.[14,21–25]

For PC, several proteomic studies have been performed since the introduction of protein profiling in 2002 by Petricoin *et al*,[26] all with different fractionation platforms and type of MS.[27–33] For example, Koopman *et al.*[34] used a surface enhanced laser desorption/ionization (SELDI)-TOF approach combined with WCX and metal affinity protein chips as the solid-phase extraction (SPE) method to discriminate between patient groups with a sensitivity of 78% and a specificity of 97%. No study has been published using a combination of WCX MB and MALDI-TOF.

For this study, a MALDI-TOF serum platform in combination with functionalized WCX MB was used to generate serum protein profiles in a first attempt to differentiate between PC patients and healthy controls in a stringent sample handling and high throughput and automated processing protocol. The obtained discriminating profile was validated in a second case–control group.

Material and methods

Patients

Blood samples were obtained from 50 patients with PC prior to surgery, and from 110 (age-and gender-matched) healthy volunteers at the outpatient clinic of the Leiden University Medical Center (LUMC), the Netherlands from October 2002 until December 2008. Healthy volunteers were partners or accompanying persons of included patients. For the validation set, blood samples were obtained from 39 patients and 75 healthy (age and gender matched) volunteers, included from January 2009 until July 2010. Patients were selected candidates for curative surgery; this meant that no patients with primary irresectable tumours were included. All surgical specimens were examined according to routine histological evaluation and the extent of the tumour spread was assessed by TNM (TNM Classification of Malignant Tumours) classification. Furthermore, the tumour marker CA-19.9 was noted if determined pre-operatively. An Elecsys CA19-9 tumour marker assay based on the monoclonal 116-NS 19-9 antibody (Roche Diagnostics GmbH, Mannheim, Germany) was used. This tumour marker has a normal reference value of 0.0–27.9 U/ml (95th percentile). Informed consent was obtained from all subjects and the study was approved by the Medical Ethical Committee of the LUMC.

Blood collection

Samples from both the calibration set and the validation set were collected and processed according a standardized protocol:[14] in short, all blood samples were drawn by antecubital venapuncture while the individuals were seated and had not been fasting prior to any invasive procedure. The samples were collected in an 8.5-cc Serum Separator Vacutainer Tube (BD Diagnostics, Plymouth, UK) and maximally within 4 h at room temperature centrifuged at 1000 *g* for 10 min.[14] The samples were then distributed into sterile 500-µl barcode labelled polypropylene aliquots (Thermo Fisher Scientific, Hudson, NH, USA) and stored at −80°C.

Sample processing

Aliquotting and storage

An overview of the processing platform of the serum samples, MALDI-TOF profiles and data is given in Fig. 1. All serum samples were thawed on ice once and randomly placed in barcode labelled racks in an 8-channel Hamilton STAR® pipetting robot (Hamilton) for automated aliquotting in 60-µl daughter tubes. The aliquots were stored at −80°C until further sample processing. The processing steps for the validation set were identical to those for the calibration set.

WCX-MB sample work up

The isolation of proteins from serum was performed using a commercially available kit based on magnetic bead purification (Bruker Daltonics, Bremen, Germany). The WCX MB were applied according to the manufacturer's instructions with further optimization to allow implementation on a 96-channel Hamilton

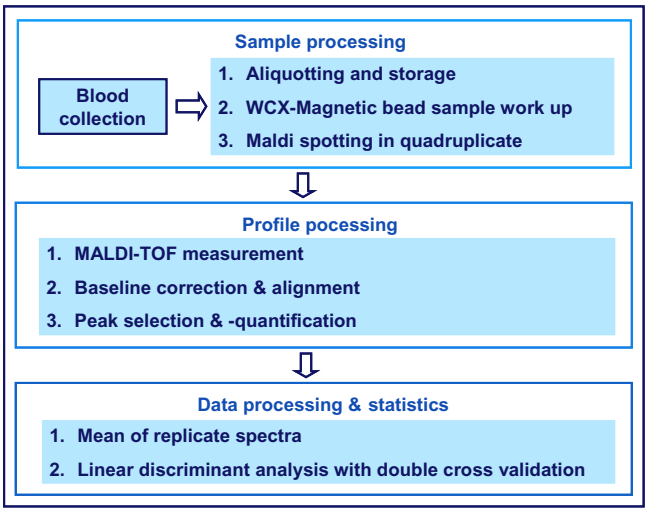

Figure 1 Overview of the used workflow model of sample, profile, data processing and statistics

Sample processing

1 Aliquotting and storage of the serum at –80°C until further processing.

2 Automated fractionation of samples with Weak Cation Exchange (WCX) magnetic beads (MB). Storage until measurement at –80°C.

3 Automated spotting in quadruplicate on a matrix-assisted laser desorption/ionization (MALDI) plate.

Profile processing

1 Measurements of MALDI plates in MALDI-time of flight (TOF).

2 Baseline correction and alignment of four profiles per samples, removal of spectra without a signal owing to a spotting failure.

3 Selection of interesting peaks and extraction of this data with Xtractor.

Data processing and statistics

1 Data analysis on mean of the remaining profiles.

2 Analysis of the profiles with linear discriminant analysis with double-cross validation.

STARplus® pipetting robot (Hamilton). Binding- and washing buffers were used from the kit. In the final pipetting steps, peptides and proteins were first eluted from the beads using a 130-mM ammoniumhydroxide solution (J.T. Baker, Deventer, the Netherlands) and then stabilized with a 3% trifluoro acetic acid solution (Sigma, St Louis, MO, USA).

MALDI spotting

Two microlitres of each stabilized eluate was transferred into a 384-well microtitration plate to carry out mixing with MALDI matrix (α-cyano-4-hydroxycinnamic acid from Bruker Daltonics, 3 mg/ml in acetone/ethanol 1:2). One microlitre of this mixture was spotted in quadruplicate onto a MALDI 600 µm AnchorChip™ plate (Bruker Daltonics).

Profile processing

MALDI-TOF measurement

MALDI-TOF mass spectra (profiles) of the peptides and proteins were obtained using a positive-ion linear mode acquisition on an Ultraflex II TOF/TOF spectrometer (Bruker Daltonics) equipped with a SCOUT ion source and controlled by the Flexcontrol 3.0 software package (Bruker Daltonics). Ions generated by the Smartbeam™ laser were accelerated to 25 kV and mass analysed from 960 to 11 024 Da. Each mass spectrum represents the sum of 20 mass spectra obtained from 60 laser shots. All unprocessed spectra were exported from the Ultraflex II in standard 8-bit binary ASCII format.

Baseline correction and alignment of profiles

For optimal data analysis, all profiles generated after sample workup with WCX MB (further referred to as WCX profiles) required baseline correction followed by alignment. First, a baseline subtraction of all profiles was performed using the baseline subtraction tool of FlexAnalysis 3.0. Second, to perform the alignment of WCX profiles from one MALDI target plate at least three peptides at different *m/z* values were essential for internal calibration. In order to compensate for the possible absence of one or two peptides in a spectrum, the following seven peptides were selected based on a manual inspection of a few spectra, namely at *m/z* 1866.1, 3158.0, 4643.6, 5903.7, 6631.1, 7765.5 and 9290.9.[22,35]

Peak selection and -quantification

Protein and/or peptide signals in WCX profiles were quantified as follows. First, based on visual inspection of the profiles, 113 peaks were selected for further analysis. To this end, a so-called reference file was compiled including a certain *m/z* window for each signal or peak. This *m/z* window reflected the peak width and varied from 5–30 Da. Three examples of the selected peaks are shown in Fig. 2. Then, the in-house developed Xtractor tool was used to determine the intensity of each user-defined peak. This open source tool generates uniform data (peak) arrays regardless of spectral content (http://ms-utils.org/Xtractor/).

MALDI-TOF profiles were exported as DAT (.dat) files, all containing *m/z* values with corresponding intensities.

Data processing and statistics

Mean of replicate spectra

The peak files generated by Xtractor were used for data analysis. The mean of the remaining profiles of the quadruplicate spots for the WCX purified samples was used. These processed profiles will be further referred to as the WCX dataset.

For each i^{th} patient, $i = 1, \ldots, n$ *a* set of spectral measurements $x^1_i = (x^1_{i1}, \ldots, x^1_{il})$ is collected from the WCX bead processed samples such that the complete data may be represented by the matrix

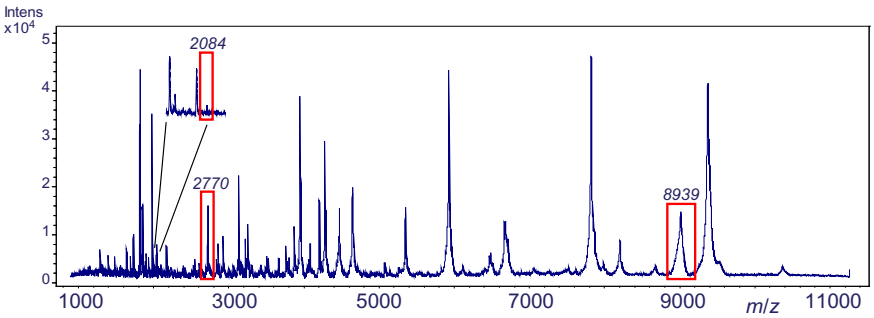

Figure 2 An example of three of the selected peaks from the Weak Cation Exchange (WCX) fractionated sampling. On the *x*-axis *m/z* values are shown, on the *y*-axis intensities. The peaks with a *m/z* of 2084, 2770 and 8393 were highlighted

$$X = \begin{pmatrix} x_1 \\ \vdots \\ x_n \end{pmatrix}$$

with $n = 160$ and $l = 113$ (calibration set) represent the dimensionality of the peak list. To complete the observed information on individuals, we have the binary case–control outcome Y which equals 1 for cases or 0 for controls[36] ($n = 50$ cases, $n = 110$ controls). The same procedure was performed for the validation set with $n = 114$ and $l = 113$ ($n = 39$ cases, $n = 75$ controls).

Linear discriminant analysis

As previously described by Mertens *et al.*[35] and de Noo *et al.*,[25] a double cross-validatory implementation of linear discriminant analysis for the calibration of a diagnostic rule based on a single (mean) spectrum per patient was performed. Each sample was assigned to the group for which the probability was highest. For each analysis error rate (Error), sensitivity, specificity and the area under the curve (AUC) were calculated. The error rates are based on sensitivity and specificity values, assuming a prior class probability of 0.5 for each group. The validation data were subsequently predicted from a single calibration of the discriminant on the calibration data (based on the observed optima from the double-cross-validatory analysis) and results were compared with known disease status.

Receiver-operating characteristic (ROC) curves with the true positive rate (sensitivity) are plotted in function of the false-positive rate (1-specificity) for different cut-off points of a parameter. Each point on the ROC curve represents a sensitivity/specificity pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal).

An independent Student's *t*-test on the case–control calibration data was performed on a selection of the most discriminating peaks observed in the calibrated discriminant model on the calibration data. A *P*-value of < 0.05 was considered as significant.

Subsequently, all analyses were repeated after an additional internal standardization performed on the calibration and validation sets separately, using the mean of all spectra and standard deviation (SD) such that the new data within each set is obtained as

$$X\text{^*\_i} = \frac{X_i - \bar{X}_i}{SD_i}$$

to minimize differences in peak intensities between the calibration set and the validation set which may be caused by batch effects. It should be noted that the above standardization does not affect calibrations or results on the calibration data (but it does for any predictive analysis, such as in double cross-validation or when predicting the validation set), as linear discrimination is invariant to the above standardization.

Results

Patients

Patient characteristics for each set are shown in Table 1. All patients were diagnosed with adenocarcinoma of the pancreas. The calibration set consisted of 50 pre-operative PC patients ($n =$ 23 male, $n = 27$ female) with a median age of 66 years (range 41–80). The validation set consisted of 39 pre-operative PC patients (17 male, 22 female), included with the same criteria as the calibration set. The median age of this group was 63 years (range 38–81).

The median age of the control group in the calibration set ($n =$ 50 male, $n = 60$ female) was 63 years (range 44–80) and the median age of the control group in the validation set ($n = 27$ male, $n = 48$ female) was 46 years (range 21–75). No significant difference in the median ages of the cases and the controls in the calibration set ($P = 0.073$) was seen. In the validation set a difference was observed with a *P*-value of <0.001.

In the calibration set, 12 (24%) patients had stage I, 24 (48%) had stage II, 5 (10%) had stage III and 9 (18%) had stage IV. In the validation set, 4 (10.3%) patients were classified as stage I, 24 (61.5%) as stage II, 2 (5.1%) as stage III and 9 (23.1%) as stage IV.

Table 1 Patient characteristics for the calibration and the validation set

	Calibration set			Validation set		
	Cases	Controls	Correct classification	Cases	Controls	Correct classification
N	50	110		39	75	
Age median, years (min – max)	65.8 (41–80)	63.0 (44–80)[a]		63.4 (38–81)	45.9 (21–75)[b]	
Male	23	50		17	27	
Female	27	60		22	48	
Diagnosis						
Adenocarcinoma	50		38 (76%)	39		29 (74%)
Stage						
IA	7		6 (86%)	2		0 (0%)
IB	5		5 (100%)	2		1 (50%)
IIA	3		2 (67%)	3		2 (75%)
IIB	21		16 (76%)[c]	21		18 (86%)
III	5		3 (60%)	2		2 (100%)
IV	9		6 (67%)	9		6 (67%)
CA19-9	26		19 (73%)	20		14 (70%)
Positive	17		12 (71%) [c]	13		11 (85%)
Negative	9		7 (78%)	7		3 (43%)

[a]$P = 0.073$.
[b]$P = 0.000$.
[c]$n = 1$ missing as a result of a low-quality profile.

In 29 patients (32.6%) the tumour appeared irresectable during surgery. In all other cases ($n = 60$) a pancreaticoduodenectomy (65.2%) or a pancreatic tail resection (2.2%) was performed.

In total, five patients (5.7%) had an increased inherited risk for developing PC. In four patients a P16-Leiden mutation (also annotated as a 19-base pair deletion of exon 2 of the CDKN2A gene) was found. One patient was a BRCA2 mutation carrier.

Statistical analysis

In total, 274 serum samples were processed with WCX MB and MALDI-TOF profiles were obtained in quadruplicate, yielding 1096 WCX profiles. Only one sample was excluded from further statistical analysis because of low-quality profiles (as a result of failed sample workup or not optimal MALDI spotting).

Linear discriminant analysis with double cross-validation resulted in an error of 0.2054, a sensitivity of 73% and a specificity of 85% with an AUC of 0.90 (Table 2) Next, we selected a number of most discriminating peaks, based on the fitted discriminant weights from a single recalibration of the linear discriminant rule, based on the observed optima from the previous double cross-validatory analysis. We selected seven peaks with m/*z* values of 2084, 2178, 2770, 2899, 3096, 8760 and 8939. In Table 3 these peaks are shown together with a two-sample *t*-test, the corresponding pooled estimate of the population standard deviation, *P*-value and confidence interval.

Subsequently, calculations after internal standardization were repeated as previously described and using the above set of selected peaks in the calibration set which gave an error rate of 0.12, a sensitivity of 84%, a specificity of 92% and an AUC of 0.932. However, this result is likely strongly biased because of the absence of any validatory analysis and the preliminary peak selection. Adding double-cross validation to this analysis to reduce the bias resulted in the final results of an error rate of 0.17, a sensitivity of 78% and a specificity of 89% with an AUC of 0.897 (highlighted in blue). Next, the standardized validation data were predicted, using the peak-selected calibration classification rule on standardized calibration data, confirming our first findings; which gave an error rate of 0.17, a sensitivity of 74%, a specificity of 91% and an AUC of 0.893 (Table 2). ROC curves of both the calibration as well as the validation set are shown in Fig. 3.

A correct classification was obtained for 38 out of 50 (76%) PC patients in the calibration set. In Table 1 these classifications are displayed per diagnosis and stage. Seventy-six per cent of the correctly classified cases were diagnosed with an early stage of PC (stage I and II). On the other hand, 29 (81%) of the 36 patients with an early stage were correctly classified. In the validation set, a correct classification was achieved for 29 of 39 (74%) patients. Twenty-one of these patients (72%) were operated upon in an early stage and 75% of the patients with an early stage were correctly classified. None of the two patients with stage 1A in the validation set were correctly classified. This concerns one male, aged 60 years, diagnosed with a grade 2, small, 17-mm adenocarcinoma of the pancreas and one female, aged 63 years, diagnosed with a grade 1, small, 5-mm adenocarcinoma of the pancreas.

Table 2 Error, sensitivity, specificity and area under the curve (AUC) data in percentages from various analyses for the calibration and the validation set

Data	Calibration set			Validation set	
	Original data (113 peaks)	Selected 7 peaks	Selected 7 peaks	Original data (113 peaks)	Selected 7 peaks
Double-cross validation	Yes	No	**Yes**	NA	**NA**
Standardized	No	Yes	**Yes**	No	**Yes**
Error %	21	12	**17**	23	**17**
Sensitivity %	73	84	**78**	59	**74**
Specificity %	85	92	**89**	95	**91**
AUC	0.895	0.932	**0.897**	0.94	**0.893**

The first column represents the double-cross validation results of the analysis on 113 peaks. In the second column the results from the standardized data are shown without double-cross validation. The third column shows the most reliable results after standardization and double-cross validation. The last two columns represent the validation data with and without standardization. Highlighted in bold are the two columns representing the definitive data of the calibration set and the validation set.
NA, not applicable.

Table 3 The seven most discriminating peaks in Dalton with a corresponding *t*-test value, standard deviation (SD), *P*-value and 95%- confidence interval

m/z value	t-test	SD	*P*-value	Confidence interval	
2084	–3.7054	0.9674	0.00029*	–0.9438	–0.2875
2178	–0.5519	0.8925	0.58179	–0.3874	0.2182
2770	–5.5234	0.8236	0.00014*	–1.0607	–0.5019
2899	0.1172	0.7440	0.90683	–0.2374	0.2674
3096	–2.7116	0.7581	0.00744*	–0.6103	–0.0959
8760	–1.2342	1.1477	0.21898	–0.6327	0.1461
8939	0.5841	1.0086	0.56002	–0.2410	0.4433

Significant peaks with a *P*-value <0.05 are marked with a *.

Serum biomarker

As shown in Table 1, in the calibration set, for 26 (52%) patients a pre-operative CA19-9 value was determined. This is compared with 20 (51%) in the validation set.

In the calibration set 17 (65.4%) patients had an elevated CA19-9 value. Nineteen (73%) patients were correctly classified with protein profiling. When protein profiling was compared with CA19-9, protein profiling correctly classified two (19 versus 17) more than CA19-9. In nine (34.6%) patients CA19-9 was not increased. Seven (78%) of these patients were correctly classified by protein profiling. In total, protein profiling gives additional information in nine (two and seven) cases.

In the validation set, 65% (n = 13) of the patients had an elevated CA19-9 value. Fourteen (70%) of the 20 patients with a known CA19-9 value were correctly classified. Protein profiling was correctly classifying one (14 versus 13) more than CA19-9. In seven (35%) patients CA19-9 was not increased. Three of these patients (43%) were correctly classified. In total, in the validation set, protein profiling gave additional information in four (one and three) cases.

Patients with increased risk

Three of the five (60%) patients with an increased risk were correctly classified with protein profiling (two with a P16-Leiden mutation, one with a BRCA2 mutation).

In three cases CA19-9 was known, two with elevated CA19-9 values. One of these patients was correctly classified with protein profiling. One patient had no increased CA19-9 value. This patient was incorrectly classified by protein profiling. Thus, protein profiling gave additional information in one case.

Discussion

The accurate and early detection of PC may result in more patients that could benefit from a pancreaticoduodenectomy and may increase the survival time. Clinical proteomics has emerged as a promising strategy to develop novel tools for biomarker strategies.[37–42]

In this study, high-resolution MS profiling of human serum was used, with the aim of detecting specific patterns present in patients with PC. Using this method, a set of seven peptides was found that differentiated PC from healthy volunteers with a sensitivity of 78% and a specificity of 89%. These results were successfully validated in an independent case–control group.

Although in several previous studies serum protein patterns were found to show a high sensitivity and specificity as an early diagnostic tool, critical notes have been made on biological variation, pre-analytical conditions and analytical reproducibility of serum protein profiles.[43] Thus, the application of proteomic spectra can only be applied in a routine clinical setting when the collection and processing of the data is subjected to stringent quality control procedures.[14,21,43] In the Leiden University Medical Center, much effort has been spent in optimizing the protocol for high throughput analysis. A completely robotized and automated procedure was used for sample handling. This protocol was tested with respect to the number of freeze–thaw

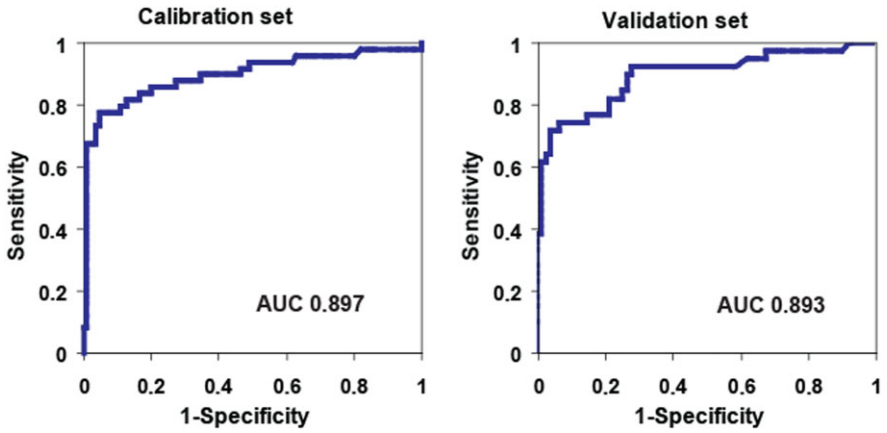

Figure 3 Receiever-opertaing characteristic (ROC) curves of the calibration and validation set after peak selection with standardization

cycles, the period between collection and serum centrifugation and reproducibility.[14]

In principle, a high-end MS setup is sensitive enough to detect almost any protein, but the true sensitivity of MS is modulated by the nature of the sample. Biological samples are typically characterized by a wide range of protein abundances, and mass spectrometers are not well equipped to deal with this wide dynamic range. Peptides do not ionize with equal efficiency, potentially putting some proteins at a disadvantage in terms of detection. This issue is further complicated by low-molecular-weight proteins and those expressed at low abundance. Sample fractionation overcomes these issues, reduces the impact of undersampling and improves the reproducibility between analyses.[13] In the present study, WCX magnetic beads were used with specific binding characteristics. These magnetic beads can be well implemented in a robotic platform for fully automated use and this automation ensures the control of each step in the extraction protocol and thus minimizes technical variability.[20] In addition, this automation in combination with the high speed of data acquisition of MALDI MS allows high-throughput analysis of thousands of samples.

In 2007, Goonetilleke *et al.* published a systematic review about CA19-9 as a diagnostic serum marker for PC. They calculated a median sensitivity of 79% and a median specificity of 82%.[44] With the present protein profiling a comparable sensitivity but a higher specificity was achieved. In 13 reports, CEA shows a mean sensitivity of 54% and a specificity of 79%.

Other newer serum biomarkers described by Goonetilleke *et al.* are carbohydrate antigen 242 (CA 242), carbohydrate antigen 50 (CA 50), SPAN-1 and sialyl-lcat-N-tetraose (DUPAN-2). These newer biomarkers perform with sensitivities of 65–83% and specificities of 80–93%. Until now, none of these biomarkers are clinically used.

In this study, CA19-9 was semi-routinely assessed with an overall sensitivity of 65% and 63%, respectively. Although this marker is not clinically used for the detection of early PC, we choose to compare our profile with this 'best currently available' marker. Comparing the protein profiling classification with CA 19-9 values resulted in an improvement for 13 patients.

In the calibration set, 29 out of 36 patients (81%) with stage I and II PC were correctly classified, whereas in the validation set early PC was correctly classified in 21 out of 28 patients (75%). Stage IA and IB differentiate between the tumour size of 2 cm. Also for higher stages, the test showed a high sensitivity and specificity. In total, this gives a sensitivity of 78% for the detection of early PC with our profile.

Nowadays, the emphasis in biomarker research has moved towards peak identification, i.e. biomarker characterization. It is convenient to first determine the diagnostic power of candidate markers before performing identification studies and further investigations into their biological role in disease mechanisms, as identification of peptide- or protein signals in a profile is not straight forward. Such efforts require specific separation- or enrichment strategies and a high MS/MS data quality for identification of endogenous species, i.e. large coverage of fragment ions. Until now, most reported identifications of serum peptides in profiles were based on SELDI enrichment chips [i.e. Immobilized Metal Affinity Capture (IMAC)[34]] or on Reversed Phase (RP)C18 solid-phase extraction (SPE) procedures[45–47] and can therefore not directly be overlaid with WCX profiles. Only a few peptide/protein identifications from WCX fractionation have been reported.[23,34]

Previously, peaks in MALDI-TOF profiles at *m/z*'s of 2084, 2178, 2770, 2899, 8769 and 8939 have been characterized as fragments of FGA-chain, Apo-CIII and Apo-CII[45] Although these *m/z* values correspond with the seven most discriminating peaks of this study, these characterizations have been performed after RPC18 purification and are therefore not completely transposable with our data. The latter peak of 8.9 kDa is also described in the literature as Human Complement C3 (P01024, Unitprot.org), but identified as a discriminating peptide after fractionation with IMAC beads. Recently, Albrethsen *et al.*[48] published an overview of

the MALDI and SELDI characterization of peptides and proteins, but these m/*z* values did not correspond exactly with the seven discriminating peaks of this study.

The present profile is not yet tested on individuals with an increased risk (smoking, chronic pancreatitis and diabetes) of developing PC or an increased inherited risk (carriers of a p16-Leiden mutation, Peutz–Jeghers syndrome, FAMMM or BRCA2 mutation carriers). This group might especially benefit from a highly sensitive and specific new biomarker. A retrospective series will be analysed and the collection of serum during surveillance of this high-risk group has been set up.

Furthermore the method should be further improved before it is clinical applicable. The first step, which was presented in this study, is that we can identify patients with pancreatic cancer from healthy controls. However, the value of the test for high-risk patients as patients with diabetes, smoking, pancreatitis and/or other contributing factors is still unknown. A next step in our research programme is to analyse the serum proteomics parameter in a group of patients with an increased risk for developing PC (smoking, chronic pancreatitis and diabetes). Furthermore the research continues to improve the cleaning up procedure using two different MB, with promising preliminary results. This could mean that sensitivity and specificity could be improved.

In conclusion, MS technology allows high throughput analysis of peptides and proteins, with accurate results and, when properly applied, with high reproducibility. Protein profiling can classify pancreatic patients from healthy volunteers based on the SPE fractionation with WCX MB. This promising new biomarker is a simple, additional test for the diagnosis of PC in clinical practice. Further research is necessary to evaluate its specificity.

Conflicts of interest

None declared.

Authors's contributions

We declare that all authors meet the following three conditions:

(1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
(2) drafting the article or revising it critically for important intellectual content; and
(3) final approval of the version to be published.

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